TISSUE CULTURE STUDIES OF ANTIBODY FORMATION

by

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INTRODUCTION

The original purpose of this thesis was to attempt to induce antibody synthesis by a cell line *in vitro*. The literature contained a number of references to antibody forming systems of short duration by cells derived from previously immunized animals. The initiation and continuation of true *in vitro* antibody synthesis had been reported by only a few individuals. In preparation for this experiment it seemed desirable to first develop technics for maintaining in a functional condition antibody forming cells and for detecting small amounts of antibody. Therefore, attempts were made to isolate from preimmunized animals, cells that would continue to synthesize antibody *in vitro* for an extended period of time.

The basic reason for undertaking such an endeavor was to eventually better understand the antibody response, the complexity of which may be coming to light at the present time. Evidence is now at hand suggesting that information stored in some complex molecule must be transferred from one cell type to another. Furthermore, the maturation or development of cells may be an important factor, as has been frequently noted in histological studies. Perhaps cellular selection, even at the genetic level, may be important. Certainly none of these phenomena are understood, and their relationship to the immune response is yet to be established.

Carrel and Ingebrigtsen (2) in 1912 reported that small pieces of guinea pig bone marrow and lymph glands cultivated in plasma clots produced hemolysins after primary, *in vitro* exposure to whole sheep red blood cells. The hemolysins removed from the culture fluid were specifically absorbed by antigen, were active only in the presence of

complement, and were not present in the fluid over unimmunized or killed cultures. This early report of apparent antibody synthesis *in vitro* was incomplete, and did not give the number of controls employed or exclude the possibility that hemolysins were present in some of the materials introduced.

Herman Ludke (10) in that same year published an independent report in the German literature stating that agglutinins to the typhoid bacillus were produced in tissue culture and in recipients following cell transfer.

In 1937 Parker (16,17) reported that agglutinins could be demonstrated in the fluid overlaying fragments of cultured rabbit spleen, if the rabbit had been sacrificed two days after the intravenous injection of guinea pig red blood cells. However, no agglutinins could be detected if the antigen was added directly to the culture. His medium consisted simply of normal rabbit serum in a balanced salt solution.

Hwon (6) in 1937 incubated fragments of rabbit spleens in a medium composed of heparinized rabbit plasma, rabbit spleen extract, and Tyrode's solution. He noted that if rabbits were exposed to diphtheria toxin at least one hour before sacrifice, antitoxin appeared in the supernatant fluid about two days later. If the antigen was added directly to the culture fluid (the animal having had no previous exposure to the antigen), no antitoxin could be detected.

Fagraeus (3) reported a paper in 1948 on antibody formation by splenic tissue cultures. This study was progressive in that it used tissue culture as a tool used in conjunction with histologic observations. She correlated antibody production with the appearance of plasma cells in the red pulp of the spleen. Therefore, the *in vitro* rates of release of antibody by two preparations (white pulp vs red pulp) were quantitated relative to each other.

Her technique of immunization consisted of subcutaneous injections of formalized _S. typhi_ organisms in agar, followed in 14 to 25 days by the intravenous injection of living organisms. She dissected the red pulp from the white pulp, and cultured small fragments of each in a medium containing 50% normal rabbit serum and modified Tyrode's solution. She found that the red pulp seemed to consistently produce higher agglutinin titers than the white. An interesting additional finding, reflecting the dynamics of antibody production, was that more antibody was formed during the second 12 hours of incubation in cultures derived from animals sacrificed 3 days after the intravenous injection of living organisms. If the spleen was cultured 5 or more days after the secondary injection, more antibody was produced _in vitro_ during the first 12 hours of incubation.

Keuning and van der Slikke (7) in 1950, and Thorbecke and Keuning (25,26) in 1953 modified the methods of Fagraeus only slightly. They extended the preparations studied to include bone marrow, liver, mesenteric lymph nodes, and thymus. They concluded that in adult rabbits secondarily immunized via the intravenous route, the spleen and bone marrow were excellent sources of antibody forming tissue. The liver apparently formed some antibody, but only in splenectomized animals. The mesenteric nodes possessed weak activity that was not enhanced by splenectomy, but the thymus was inactive. They also presented evidence of a parallelism between antibody synthesis and gamma globulin production _in vitro_ as measured by the precipitin reaction.

In the experiments reported above measurements of antibody activity were based on titration procedures using serial two-fold dilutions, which are semi-quantitative and which may be non-specific.

Fagraeus computed "antibody units" per gram of tissue, which was

simply the reciprocal of the highest dilution of culture fluid-giving agglutination divided by the weight of the tissue used. Controls consisted of titrations of extracts from the same amount of unincubated tissue, that were stored for one to four days at 4 degrees C, after extraction.

An additional control included one in which lethal amounts of toluol was added to some of the cultures, resulting in the complete suppression of agglutinatin formation. For completeness the agglutinins should have been tested for their stability during extraction and storage, for their resistance to adverse effects by toluol, and for their specificity. This is to insure that the difference between the experimental and control titers was not due to a difference in treatment. For these reasons the data in Fagraeus' paper are open to question.

Thorbecke and Keuning compared the titers of saline extracts of equal quantities of non-incubated (control) tissue with the titer of incubated tissue extracted by the same methods as the control in the tissue's own culture fluid 24-72 hours later. A "production index" was calculated by dividing the reciprocal of the control titer into the difference between the reciprocals of the culture titer and the control titer. One of the most common objections to study of this type is that preformed antibody is being "released" but not synthesized under the conditions of incubation. Several other explanations for a "production index" of one or greater can be considered besides an increase in antibody concentration in the cultured system.

Perhaps the concentration of antibody decreased in the control extracts while being stored in the refrigerator, especially since proteolytic enzymes may have been released during maceration. The test culture fluids may have acquired some nonspecific agglutinating factor

such as a low pH, or the release of nonspecific agglutinatins following incubation. Human factors are also present since the "last tube showing agglutination" is not a sharp end point. It should also be noted that a "production index" of two would represent more antibody synthesized when the control titer was high than when it was low, for purely mathematical reasons. No statistical tests of significance have been applied to any of the studies so far mentioned.

Mountain (14) in 1955 investigated the effects of a wide variety of substances on the synthesis of H agglutinins by cultures of minced rabbit spleen. She carefully controlled the stability of antibody under the conditions of incubation (including exposure to substances tested), and performed most of her titrations in triplicate. She also gave an approximate indication of the probability of chance differences between control and experimental values.

She found that mechanically disrupting the cells by grinding destroyed much of their capacity to form antibody. 10^{-4} M sodium cyanide, 10^{-3} M magnesium sulfate, 10^{-3} M ferric chloride, and 10^{-4} M cupric sulfate all had an inhibiting effect on antibody synthesis _in vitro_. The addition of purine and pyrimidine bases, certain vitamins and vitamin analogs did not alter _in vitro_ synthesis. The addition of ten amino acids (cystine, cysteine, and glutathione were not included) at a combined concentration of 30mM inhibited antibody synthesis; but at a concentration of 15mM the hydrolysate of casein did not influence the titer. Cysteine, ascorbic acid, and reduced glutathione at concentration of 15mM or higher inhibited agglutinin formation. Cortisone inhibited synthesis at high concentration, whereas insulin, thyroxine, or adrenaline had no detectable effect. These findings would suggest that

the concentration of various nutrients including amino acids is of great importance. Mountain maintained her cultures for periods up to three days, after which time the titers declined. In fact, antibody synthesis *in vitro* for periods longer than 72 hours had not yet been claimed by any of the investigators so far mentioned.

Stavitsky (20) in 1955 reported the *in vitro* synthesis of antitoxin by fragments of lymph node and spleen from previously unimmunized rabbits. He attempted to further overcome the objection that the *in vitro* rise in agglutination titer was due to the carrying over of antibody formed *in vivo* by performing multiple extraction procedures on fresh immune tissue to make certain that all the preformed antibody was measured and accounted for. Although such efforts must necessarily fall short of absolute proof, they do lend support to the concept that antibody continues to be formed for at least a short time after the tissues are removed from the animal. The relative antibody synthesising activity of different tissues was consistent with most previous studies. Synthesis was inhibited by heat and cyanide, as well as by other reagents.

Although antibody production probably did occur in Stavitsky's experiments, a critical analysis of the methods of detection is in order. In his early *in vitro* studies, he employed a modification of Boyden's tannic acid hemagglutination technique. *In vitro* synthesis was quantitated in "hemagglutinating units" which were calculated by adding the reciprocal of the culture medium titer to the tissue titer extracted with saline, and then subtracting the titer of the same amount of tissue which had been extracted at the time of initiation of the cultures. This is essentially the same as dividing a sample into two volumes, titrating each, and adding the reciprocals together. Therefore, Stavitsky's values are too high for purely physical reasons.

In Stavitsky's experiments there was a random variation between the weight of the tissues used for base-line extraction and the weight of the tissues incubated. In addition, in a non-homogeneous tissue (such as spleen), some fragments might have more preformed antibody or actual antibody-producing cells. Therefore, there is danger of disregarding the experiments in which the "net antibody production" fails to attain an arbitrary number of "hemagglutinating units," while accepting those that may have reached this arbitrary number by chance alone. For these reasons then, Stavitsky's early results must be accepted with caution.

In later work Stavitsky and Wolf (21,28) found that altering the medium could facilitate antibody synthesis _in vitro_, and that radioisotope labeled amino acid incorporation was a more sensitive detection method. A new medium (W medium) was found to be superior to medium 199 or medium V-614. One of the most distinctive features of W medium was the low total amino acid concentration of about 1.3 mM. Medium 199 has a total amino acid concentration of about 8.5 mM. The addition of more than 5% serum was inhibitory in cultures carried only 24 hours. Dialyzed serum, serum ultrafiltrate, chick embryo extract, purines, pyrimidines, vitamins, coenzymes, lipids, and carbohydrates did not enhance antibody formation.

The most extensive study of antibody synthesis has been carried out by Stevens and McKenna (24). In 1957 (11) they reported _in vitro_ antibody synthesis by diced spleen fragments obtained from previously immunized rabbits in Trowell's medium. Using the tanned red blood cell technique, hemagglutinin titers indicative of antibody synthesis appeared in vitro even if the spleen was removed as early as 10 minutes after the intravenous injection of the bovine serum albumin antigen. If endotoxin

was given 24 hours before the antigen, the subsequent *in vitro* titers were increased about eight fold. This extreme enhancement by endotoxin given in this time relationship is somewhat at variance with other studies of the biologic properties of endotoxin (8). Subsequently, Stevens and McKenna reported that if endotoxin was injected 24 hours before the sacrifice of otherwise normal animals, the synthesis of antibody could be initiated *in vitro* if these diced normal spleens were incubated with antigen.

The second major finding of McKenna and Stevens (12) was that splenic, bone marrow, and peritoneal exudate cells maintained in monolayer culture produced antibody-like substances after both *in vivo* and *in vitro*, and after entirely *in vitro* antigenic stimulation, and that descendants of these cells continued this function after proliferation and subculture for as long as four weeks. Fishman and Steral (5,23) were unable to repeat Stevens and McKenna's work.

Ogata et al (15), Askonas and Humphery (1), Steiner and Anker (22), and La Via et al (9), have demonstrated radioisotope incorporation during antibody synthesis by lymph node or splenic cells *in vitro*. These studies validate other short term studies using other detection methods. Ogata's group also demonstrated isotope incorporation into antibody, presumably during synthesis, by cell-free preparations containing microsomes.

Michaelides (13) in 1957 reported antitoxin synthesis by rabbit lymph node cells for as long as 26 days in roller tube culture. In addition, the cells cultured by this technique were able to sustain an *in vitro* anamnestic response. Unfortunately this work has only been given in abstract form, but a series of papers on this subject are to be

published. This study is of great significance in that it appears to confirm that long-term in vitro synthesis is indeed possible. Unfortunately, however, failure to confirm these observations in tissue culture using mouse spleens have been reported by Sercarz and Coons (19).

In 1959 and 1961 Fishman (4,5) reported the initiation of primary antibody synthesis in vitro with rat tissues. According to his thesis the antigen must first be acted upon by phagocytic macrophage cells which, in turn, release a substance capable of specifically stimulating cells teased from lymph nodes to produce antibody. Macrophages were obtained by injecting rats intraperitoneally with broth; the exudates were collected two days later. The antigen was then placed in contact with the washed macrophages, incubated, homogenized, and passed through a sterilizing filter. This filtrate was capable of stimulating rat lymph node cells to produce antibody specific to the antigen introduced, and this synthesis continued from the fifth day of incubation until about the twelfth day.

Two antigens were used in the system, T_2 phage and hemocyanin. When T_2 phage was employed, the specific neutralizing activity of the tissue culture fluids was detected by concentrating what was believed to be the gamma globulin present with sulfate. T_2 phage was incubated in a solution of the "antibody" concentrate, and then plated with indicator bacteria. When hemocyanin was employed, the tissue culture fluids were concentrated similarly; and the activity of the concentrate was detected by the hemagglutination of sensitized tanned red blood cells.

The controls employed included cultures of lymph node cells to which antigen alone and macrophage extract alone were added. Phage neutralization was noted only in the concentrates from cultures exposed

to the extract from macrophages incubated with antigen, and this occurred in only one out of five of the phage experiments. Tissue culture fluids that were active against the T_2 phage had no activity against phages T_1 or T_5, and those active against hemocyanin appeared to be similarly specific. The phage neutralising activity of the concentrate was destroyed by serum from rabbits immunized with rat gamma globulin.

Since the number of experiments performed is not given, and no statistical analysis was made, it is impossible to evaluate the probability that phage neutralizing material found in one out of five of the experimental cultures was not due to chance alone. Insufficient data were also given for the hemocyanin experiments. However, the use of two antigens, in addition to the fact that the above work was substantiated by transferring the variously treated lymph node cells to chick embryos, lends support to Fishman's thesis. His studies have not as yet been confirmed.

A very striking finding in Fishman's work was that streptomycin and ribonuclease both appeared to inhibit antibody synthesis, possibly by interfering with the transfer of information from the macrophages to the lymph node cells.

MATERIAL AND METHODS

Initially the spleens of mice were employed in an effort to obtain immunologically active cells for tissue culture. NIH mice were sacrificed by disjointing the neck, and their spleens were removed aseptically to a cytosieve. A cell suspension was obtained by adding about 5 ml of Hanks' solution and passing the spleen through the sieve. Smears of this suspension stained with Wright's stain revealed almost entirely lymphocytes and red blood cells, with many disintegrated cells. The cells were then centrifuged and resuspended in 8 ml of medium 199 containing 10% human serum and 100 units of penicillin per ml, or in medium 199 with 100 units of penicillin per milliliter but without serum. Two milliliters of this suspension was then added to 15x1.6 cm culture tubes with a screw top cap. The gas phase over the cultures was either 95% oxygen and 5% carbon dioxide or air. The tubes were sealed and incubated at 37 degrees C. All of these cells remained as small refractile spheres that failed to attach to the glass by 48 hours. Smears of these cells revealed pyknotic nuclei with very little cytoplasm.

Cultures of Minced Tissue on Tantalum Gauze

Because these simple cultures of cell suspensions failed to give the desired cultures, a modification of a method reported by Trowell (27) was attempted. Tantalum gauze was bent into the form of small tables 30x30 mm and 4 mm high by bending two opposite edges of a rectangular piece of gauze to serve as legs. Lens paper (Scientific Supplies Co.) was tested for toxicity by culturing fragments as large as 1.9 square centimeters in Leighton tube cultures of human amnion cells supplied by

Dr. Alan Meyer, and found to be non-toxic for these cells. The tantalum gauze tables were placed in 50 mm petri dishes, and a piece of lens paper tha t approximated the size of the flat surface of the gauze was placed on top. They were then sterilized as a unit. Medium was prepared by adding 10% human serum to medium 199 with 200 units of penicillin per milliter.

Para-aortic lymph nodes and spleens were removed aseptically from 7-month-old white rats. The spleen was minced into fragments 2 to 3 mm square, and the larger nodes were cut in two although most fragments were larger than 3 mm. Fragments of the respective tissues were placed on the lens paper and sufficient medium was added to raise the surface of medium to the tantalum gauze mesh. The petri dishes were then placed in desiccator jars containing a small amount of physiological saline for equilibration. The jars were then flushed with 95% O_2 and 5% CO_2, sealed, and incubated at 37 degrees C.

Similar experiments were performed using lymph nodes and spleen from 3 Kg stock rabbits with the following modifications in media. Insulin was twice recrystalized at pH 5.1 to 5.3.

Medium I - 20% medium 199, 80% Tyrode's solution, 200 units penicillin/ml, 5 mg insulin/100ml

Medium II - 100% medium 199, 200 units penicillin/ml, 5 mg insulin/100 ml

Medium III - 50% medium 199, 200 units penicillin/ml, 5 mg insulin/100 ml

Medium IV - 100% medium 199, 5 mg insulin/100 ml, 2.4 mg cocarboxylase/100 ml, 200 units penicillin/ml

<u>Leighton Tube Cultures</u>

I. Animals.

White rabbits were obtained either through the animal department or from the Wilson rabbitry (Beaverton) and were maintained on pellets and water *ad libitum*. They varied from two to six pounds depending on their maturity. Some of the stock rabbits developed a chronic bronchitis or bronchopneumonia following repeated bleeding or during immunization. Bacteriological cultures revealed *Bordetella bronchiseptica*. However, most animals with gross evidence of disease were excluded.

II. Antigen.

Salmonella senftenberg was inoculated centrally into petri dishes containing motility medium approximately one-half cm deep. After several hours of incubation the most motile organisms from the visible edge of outgrowth were selected, and a single loop was inoculated into 500 ml volumes of clear extract broth. The broth were incubated 24 hours and the concentration of organisms was determined by colony counts on serial dilutions. After samples for counting were removed, sufficient formaldehyde was added to the broth cultures to bring the concentration to 0.3%. There were approximately 4×10^8 organisms/ml in the extract broth at the time formaldehyde was added. After at least 48 hours in the cold the broth was cultured to insure sterility. Prior to use, the formalized organisms were collected by centrifugation, washed three times in one-fourth the volume of the broth of normal saline, and resuspended in one-fourth the original volume of broth.

III. Media.

Medium 199 was used as the basis for all media. In all instances 2.4 mg of cocarboxylase per 100 ml, 5 mg of insulin per 100 ml, and 200 units of penicillin per ml were added. The insulin was twice recrystalized in all experiments except the cultures of rabbit number 5. The

composition of each medium is given in Table 1.

IV. Immunization and Collection of Serum.

All injections except the final injection of rabbit number 1 were given into the marginal ear vein. Only five rabbits received a single injection, the others receiving multiple injections varying from 0.2 ml to 1.0 ml as noted in Table 1. Volumes of antigen as large as 1.0 ml seemed to predispose to respiratory infection.

In experiments using autologous serum the animals were bled by weekly cardiac punctures and rested at least 14 days prior to immunization.

V. Tissue culture.

Rabbits were anesthetized with 0.8 to 1.2 ml of veterinary Nembutal (R) intravenously. The abdomen was shaved, opened aseptically by layers, and the spleen removed. After removing any remaining fat the spleens were placed in a petri dish kept on ice containing 30 ml of Tyrode's solution with 10% $NaHCO_3$ added as needed to keep the pH about 7. The tissue was then gently teased apart by the use of large needles, and stirred slowly for 3 to 5 minutes. Hemocytometer counts of the resulting suspension were performed, but were considered to be inaccurate because of the presence of clumps of cells. However, such counts consistently gave about 8000 nucleated cells/cu mm. Smears stained with Wright's stain contained most of the cell types present in direct imprints of splenic tissue. Differential counts were not attempted.

Although varying amounts of the suspension was tried, 0.5 ml was found to be the most satisfactory in Leighton tube cultures. The cells were allowed to settle for about one hour, and 2 ml of medium was added. The tubes were sealed with _cafe au lait_ stoppers. In later experiments 1.0x3.5 cm cover slips were placed in the flat area of the tubes prior to inoculation, so that they could be subsequently removed and stained

TABLE 1

Rabbit #	Antigen (ml)/ Injection	Days of Injection	Day of Sacrifice	Composition of Media
1	1.0	1,2,4,7	11	Medium A 50% Tyrode's solution 50% M-199 Insulin, cocarboxylase, penicillin Medium B M-199 Insulin, cocarboxylase, penicillin Medium C 20% autologous serum 80% M-199 Insulin, cocarboxylase, penicillin
2	0.5	1,2,3,8	10	Medium C
5	0.5	1,2,4,6	8	Medium D 90% M-199 10% autologous serum Insulin, cocarboxylase, penicillin
11	0.5	1,2,3,16	17	Medium E 75% M-199 25% autologous serum Insulin, cocarboxylase, penicillin

TABLE 1 (continued)

Rabbit #	Antigen (ml)/ Injection	Days of Injection	Day of Sacrifice	Composition of Media
20	0.8	1	3	Medium F same as C using homologous serum
21	0.8	1	3	Medium F
22	0.8	1	7	Medium F
23	0.8	1	7	Medium F
26	0.2	1,2,3,8	11	Medium F
27	0.2	1,2,3,8	12	Medium F
28	0.2	1,2,3,8,40, 41, 42, 47	52	Medium F
29	0.2	1,2,3,8,40, 41, 42, 47	53	Medium F
32	0.2	1,2,3,7,40,41, 42,43,47,57	57	Medium F
33	0.2	1,2,3,7,40,41, 42,43,47,57	60	Medium F

with Giemsa stain.

VI. Titrations.

In most experiments 0.5 ml volumes of the supernatent medium was removed and replaced at regular intervals. If significant numbers of red blood cells and/or unattached nucleated cells were present in the fluid to be titrated, they were removed by centrifugation. Two-fold dilutions of the fluid to be titrated, beginning with a dilution of $\frac{1}{2}$, were set up using normal saline as diluent.

With the cultures of rabbit 1, the medium was entirely withdrawn each time a titration was performed. This proved to be impractical since it was difficult to withdraw all the medium without disturbing the cells, and too few titrations and media changes resulted. The cells degenerated in this experiment after the tenth day probably because of the infrequent changes of medium.

A saline control, a standard anti-S. seftenberg serum control, and a medium control were ordinarily used with each titration. Unfortunately, the medium control was omitted in the titration of the culture of rabbit 1. The standard anti-sera consistently were within one serial dilution of their previously determined value.

The Stability of Agglutinins in Tissue Culture

To determine the stability of agglutinins to S. seftenberg in the in vitro system, splenic tissue from a normal rabbit was cultured by the Leighton tube method described above. Medium F with sufficient antiserum added to give a titer between 1/32 and 1/64 was used. Samples removed at the time of culture, 24 and 72 hours later all agglutinated the antigen weakly at a dilution of 1/64.

Addition of Antigen In Vitro

Antigen was collected, washed three times with one-fourth the broth

volume of sterile, normal saline, and resuspended in one-fourth the broth volume of saline. It was then diluted serially as far as $2x10^{-6}$ of the resuspended concentration. Twenty-day-old Leighton tube cultures derived from the spleen of rabbit 11 then received 0.1 ml of the diluted antigen. Tube #2 received the 10^{-3} dilution; tube #3, the 10^{-4} dilution; tube #5, the 10^{-5} dilution; and tube #6, the 10^{-6} dilution. Titrations were performed one and four days later.

Duplicate sets of 19-day-old cultures from rabbit 26 and duplicate set of 20-day-old cultures from rabbit 27 each received 0.1 ml of antigen diluted to 10^{-4}, 10^{-5}, 10^{-6}, and $2x10^{-6}$. Titrations were performed 5, 8, 14, and 16 days later. Two tubes of 6-day cultures from rabbit 28 and 2 tubes of 7-day cultures from rabbit 29 received 0.1 ml of antigen diluted to 10^{-2}, and titrations were performed 1, 4, 6, and 8 days later.

Cultivation of Spleen Fragments

Pinkel (18) reported the successful culture of spleen fragments for as long as 62 days. Between 15 and 30 days of culture regeneration of the lymphoid follicles occurred so that by 30 days lymphatic tissue predominated. It was of interest to try this technique as a method of obtaining surviving antibody-forming cells *in vitro*.

Teflon rings 1.6 cm in diameter obtained from Dr. Pinkel were placed in 10 ml beakers, which were then sealed in foil and sterilized. One ml of medium was then placed in the beaker. A sterile piece of lens paper 1.8 cm in diameter and moistened in medium was placed on top of the Teflon ring so that air bubbles were not entrapped. A fragment about 2x3 mm of the spleen to be cultured was placed on the lens paper, and the cultures were sealed and incubated. The medium was partially exchanged

every three days by removing 0.5 ml from the side of the Teflon ring and adding the same amount of fresh medium. The medium consisted of 80% medium 199, 20% calf serum, 100 units penicillin per ml, and 50 micrograms of streptomycin per ml.

A total of eight mice were injected intraperitoneally with 0.3 ml of the antigen prepared as described. Four of these mice were used for tissue culture 4 days later. The remaining 4 mice were bled on the day of culture and 3 days later as controls. Four uninjected mice, whose spleens were cultured at the same time as the experimental mice, were also used as controls. Each mouse was anesthetized with 0.3 ml of a 1.5 dilution of veterinary Nembutal injected intraperitoneally on the day of culture. All mice were then bled from the infra-orbital sinus. The spleens to be cultured were removed aseptically, and a suitable fragment excised for culture.

RESULTS

I. Microscopic Examination of Cultures

Culture of cytosieve suspension. As has been mentioned, the cytosieve suspension of cells showed loss of cytoplasm and failed to survive satisfactorily in tissue culture. Because cell suspensions prepared by teasing apart the tissues did survive and proliferate in tissue culture, it seems likely that damage to the cells was responsible for this failure.

Fragments cultured on tantalum gauge. Smears made of the fragments after one day contained a definite increase in free unorganized debris and disintegrated cells over smears made at the time of culture. The cells present were mostly small lymphocytes, but large lymphocytes and some reticular cells were present. Permanent sections of tissue after four days demonstrated a definite granularity of cell cytoplasm and swelling of the connective tissue.

Permanent sections after seven days of culture showed liquefaction in the center of the tissue. Cell nuclei were vesicular and many had fragmented. The cytoplasm was granular and confluent with eosinophilic material that was distributed throughout the section. Fibrocytes in the trabeculae and capsule appeared quite intact, but most of the cells of the lymphatic series appeared to have undergone abnormal degenerative change. This type of preparation was not, therefore, considered suitable for in vitro antibody synthesis.

Leighton tube cultures. Suspensions prepared by teasing the tissue apart contained both free cells and clumps of cells of various sizes. There was attachment to the glass as early as 24 hours after initiation of the

cultures. The free cells settled and attached at random over the surface. Cells that apparently migrated from within the clumps of cells soon surrounded the clumps. This phase of attachment continued for about five days. Most cells during this phase were round or had but a single large cytoplasmic process similar to a "hand mirror." The nucleus appeared small because the cytoplasm was flattened against the glass. Plasma cells, although searched for, could not be distinguished. However, the maturity of cells could be much more easily discerned, and a gradation from large cells with prominent nucleoli and fine chromatin to small cells with clumped chromatin were seen. Pyknotic nuclei were seldom observed, perhaps because degenerating cells separated from the glass. Mitotic figures were seen only rarely during the first few days of culture.

After five days an underlying process of "selection" was evident. The number of mature cells seemed to be decreasing, and intermediate cell types disappeared. Cells with fine chromatin and prominent nucleoli began to develope pointed processes. Mitotic figures became increasingly prominent among these immature cells. Multinucleated cells, similar to Langhans giant cells, appeared. The immature cells continued to proliferate and could be subcultured by scraping them from the glass and dividing them into two or more subcultures. Although these immature cells were capable of engulfing formalized bacteria, the addition of bacteria did not stimulate maturation.

There were appreciably fewer cells attached to the glass after two days and a shortening of the period of time in which the cells continued to attach in media A and B. There was no detectable difference between the other media used.

Teflon ring cultures. Permanent sections of these cultures for the first few days were not greatly different than those of fragments cultured on tantalum gause. The initial degenerative changes occurred within the first 15 days, and the proportion of debris to intact cells then decreased. Mitotic figures were not observed, and there was no positive evidence that the hoped for "repopulation" accounted for the improved appearance by the 35th day. However, the healthy appearance of cells with small or no nucleoli and moderately clumped chromatin was encouraging. Unfortunately the technician could not section the lens paper, and the suggestion of outgrowth into the lens paper seen grossly was not substantiated microscopically.

II. Results of Titrations

Leighton tube cultures. Table 2 summarizes the results of the titrations of those Leighton tube cultures which demonstrated at least moderately good attachment and growth, and which were derived from healthy animals given multiple injections of antigen.

The cultures from rabbit #1 appear to demonstrate rising titers during the period of incubation. However, since there were no control titers of the media added, the possibility of accidental addition of exogenous agglutinins cannot be excluded. The suggestive evidence of this experiment encouraged further effort along this line.

Cultures from rabbits 2, 28, 29, and 32 demonstrated a modest rise in titer by the third day and in none of the experiments in table 2 did the early titers fall below the 0-day titer. This is within the variation of the method of measurement, but is suggestive of very early synthesis.

Certainly there is no evidence that sustained antibody synthesis

took place. The rate of decline of the titers is consistent with a loss of 25% of the agglutinins with each medium exchange after the brief rise noted above.

Rabbits 20, 21, 22, and 23 (not included in the table) received only a single injection of antigen. The resultant cultures were not associated with any *in vitro* agglutinins even though the rabbits' serum titers were within the same range as those that received four smaller injections of antigen over seven days.

Teflon ring cultures. There were no detectable agglutinins in the Teflon ring cultures. The control mice had serum titers from 1/64 to 1/512 on the day of culture, and from 1/256 to 1/1024 three days later.

Addition of antigen in vitro. The addition of antigen as described under materials and methods was not associated with the presence of agglutinins either at the time of or subsequent to the addition of antigen in the cultures of rabbits 11, 26, and 27. The six-and seven-day-old cultures from rabbits 28 and 29 had titers of 1/16 and 1/32 at the time the antigen was added. As medium was exchanged on subsequent days, these titers fell in close correspondence to the control tubes, and on the eighth day after antigen was added they were 1/4 and 1/8.

TABLE 2

Reciprocal of Titers by Day of Culture

Rabbit #1

Tube #	Day of Culture 0*	1	4	10
1	@	32	256	512
2		32	256	1024
3		@	256	ND
4		@	4	ND
5		@	ND	ND
6		ND	ND	1024
7		ND	ND	512

Rabbit #2

Tube #	Day of Culture 0*	1	2	3	4	5
1	ND	16	32	32	16	8
2		16	16	32	16	4
3		8	8	8	8	4
4		8	8	16	8	4
5		ND	16	ND	16	4
6		ND	4	ND	@	ND
7		ND	64	ND	16	4
8		ND	16	ND	4	ND
9		ND	ND	64	ND	ND
10		ND	ND	32	ND	ND
11		ND	ND	8	ND	ND
12		ND	ND	32	ND	ND
13		ND	ND	ND	32	ND
14		ND	ND	ND	32	ND
15		ND	ND	ND	@	ND
16		ND	ND	ND	@	ND
17		ND	ND	ND	ND	4
18		ND	ND	ND	ND	4
19		ND	ND	ND	ND	8
20		ND	ND	ND	ND	4
Mean of tubes at first media exchange		12	25	34	8	5

* Titer of fluid from cell suspension x 4 (for dilution with medium).

@ No agglutination at dilution of ½.

ND Not done.

TABLE 2 (Continued)

Reciprocal of Titers by Day of Culture

Rabbit #5

Tube #	Day of Culture		
	0*	2	5
1	2	2	8
2		2	4
3		2	2
4		2	4
Mean		2	4.5

Rabbit #11

Tube #	Day of Culture			
	0*	2	4	9
1	4	8	2	ND
2		8	2	@
3		8	2	@
4		8	4	2
5		4	8	4
6		4	2	@
Mean		6.6	3.3	

Rabbit #26

Tube #	Day of Culture				
	0*	3	6	8	11
Pool of all tubes (1-10)	4	4	4	4	2

Rabbit #27

Tube #	Day of Culture				
	0*	2	5	8	11
Pool of all tubes (1-10)	4	8	8	8	4

* Titer of fluid from cell suspension x 4 (for dilution with medium).

@ No agglutination at dilution of $\frac{1}{2}$.

ND Not done.

TABLE 2 (Continued)

Reciprocal of Titers by Day of Culture

Rabbit #28

Tube #	Day of Culture						
	0*	2	4	6	8	11	14
1	16	16	32	32	16	4	4
2		16	32	16	16	8	4
3		16	16	32	16	8	4
4		32	32	32	16	4	4
5		32	32	32	16	4	8
6		16	16	32	16	8	4
7		64	32	32	16	8	8
8		64	16	16	16	8	4
9		32	32	8	16	4	8
10		64	64	32	16	8	8
Mean		35	31.4	26.4	16	6.4	5.6

Rabbit #29

Tube #	Day of Culture					
	0#	3	5	7	10	13
1	32	64	32	32	32	8
2		128	32	32	32	8
3		32	32	32	32	8
4		32	32	32	32	16
5		32	32	64	32	16
6		32	32	64	32	16
7		64	4	32	32	16
8		64	32	32	32	32
9		64	32	64	32	8
10		128	32	32	ND	8
Mean		64	29.2	41.6	32	13.6

* Titer of fluid from cell suspension x 4 (for dilution with medium).

\# No agglutination at dilution of $\frac{1}{2}$.

ND Not done.

TABLE 2 (Continued)

Reciprocal of Titers by Day of Culture

Rabbit #32

Tube #	Day of Culture			
	0*	2	5	8
1&	32	32	32	ND
2&		16	16	8
3&		32	32	8
4&		32	32	32
5&		32	16	ND
6&		64	64	ND
7&		64	32	ND
8&		64	32	ND
12		64	32	ND
13		32	32	16
Mean		43.2	32	16

Rabbit #33

Tube #	Day of Culture			
	0*	2	4	8
3&	32	32	32	32
4&		32	32	16
5&		32	32	32
6&		32	32	ND
7&		32	32	ND
8&		32	16	ND
9&		32	32	ND
11		64	16	16
13		32	32	32
14		32	64	32
Mean		35.2	32	26.7

* Titer of fluid from cell suspension x 4 (for dilution with medium).

@ No agglutination at dilution of ½.

ND Not done.

& 3.5% sterile extract of normal spleen added to these tubes.

DISCUSSION

At this time there is substantial evidence in the literature that tissues from recently immunized animals continue to synthesize antibody *in vitro* for about three days. Although the earlier reports on this subject necessarily employed indirect detection techniques, radioisotope incorporation studies have been well adapted to confirm this phenomenon. Prolonged antibody synthesis *in vitro*, and antigenic stimulation *in vitro* have not been so well established. Indeed, Stevens and McKenna's reports using endotoxin have not been substantiated, and may be considered doubtful. Reports of prolonged antibody synthesis (one month) by tissues from immunized animals have thus far been published only in abstract form (13). Confirmatory work still needs to be done on clarifying the role of macrophages in permitting an *in vitro* antibody response (4,5).

The results of this thesis represent the examination of different exploratory techniques of tissue culture that might be useful in prolonged studies of antibody synthesis and for maintaining cells capable of responding to antigenic stimulation. The preparation of the tissues for culture was seen to be an important factor. The gentle teasing apart of organs such as spleen seems to be effective for preparing monolayer cultures. Small clumps of cells at the initiation of monolayer cultures appears to allow the migration of cells onto the glass with a minimum of disruption. By inference, the method by which fragments are prepared for organ culture should also be given careful consideration.

In vivo antigenic stimulation divided over several days might be more effective in obtaining a greater concentration of antibody-producing cells than giving all the antigen in a single injection. This is suggested by

the absence of agglutinins after the initiation of tissue culture in rabbits 20, 21, 22, and 23. However, since early antibody synthesis was not proven in animals given four injections over 7 or 8 days, this possibility remains open. Definite secondary stimulation (rabbits 28, 29, 32, and 33) was no more effective than primary stimulation in producing evidence of prolonged antibody formation in vitro. Thus, although the second- and third-day titers were higher, the rate of decline was the same. (See table 2.)

A radically new kind of tissue culture technique would have to be devised before tissue cultures are attained that undergo no changes in tissue organization and cellular morphology. The monolayer cultures studied here were associated with a loss of cells of intermediate maturity, which is of great significance in view of the likelihood that the antibody response is associated not only with mitosis but with maturation of cells. Immature cell forms proliferated readily, but did not undergo maturation when stimulated with particulate antigen. It was concluded that the histological appearance of these cultures was consistent with more prolonged antibody synthesis than was actually found by titration, but may not have been consistent with in vitro stimulation. In fact, the addition of multiple concentrations of antigen did not induce antibody formation in these cultures. Of course, prolonged antibody synthesis also may be dependent upon constant replenishing of the antibody synthesizing cells by maturation.

The significance of giant cells is difficult to evaluate. It appeared as though they formed by coalition of mature cells (monocytes?). Perhaps they demonstrated a reaction seen in vivo, as in the formation of Langhan's giant cells. The appearance of multinucleated cells, however,

is one of the phenomena seen in established cell lines under abnormal conditions. Whatever the genesis of these cells, they were consistently present and represented an integral part of these cultures.

In the organ culture experiments, early tissue necrosis was prominent. After 15 days in culture the proportion of necrotic tissue decreased, and the presence of intact cells at 35 days was encouraging. However, the presence of "repopulation" in the Teflon (R) ring cultures was not substantiated by the presence of mitosis, indicating that _in vitro_ antigenic stimulation may not be feasible. No evidence of antibody synthesis even for a short period of time was noted.

Because of limitations of time made more severe by infection in many of the animals, more questions arose than could be investigated. It is possible that necrosis resulted from the manner in which the fragment for organ culture was excised even though it was performed by two sharp, fine scissors. This represents an easily accessible variable that should be investigated. A methodical search of different animal species, media, and antigens, as well as tissue culture techniques, is in order. Mouse lung tissue, rabbit peritoneal exudate cells, and rabbit lymph nodes were used in addition to the tissues mentioned, but have thus far been given an inadequate trial.

SUMMARY

The data show that tissues cultured by the techniques described do not synthesize antibody for prolonged periods of time except perhaps at a very low level. Monolayer cultures may have synthesized antibody for the first one to three days of culture. Attempts to induce antibody synthesis by adding antigen *in vitro* were also unsuccessful.

Histological changes in the cultures are described. An increase in the number of attached cells of varying maturity was noted for the first five days, followed by a decrease in the number of cells of intermediate maturity. Cells with primitive characteristics proliferated readily. It was concluded that these cultures were histologically consistent with longer antibody synthesis than the titrations suggested. The absence of evidence of maturation was taken to indicate that *in vitro* antigenic stimulation was not probable.

Organ cultures were maintained as long as 35 days, but no marked proliferation or repopulation was demonstrated. Prolonged *in vitro* synthesis of antibody may be possible with refinement of this technique after *in vivo* induction, but total *in vitro* induction of antibody synthesis is unlikely.

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APPENDIX

Titration Techniques

In general titrations were performed as follows. One half ml of medium was removed from the cultures to be tested. Cellular material was removed from the specimen if necessary by centrifugation after diluting the specimen in 0.5 ml of normal saline. Further 2 fold dilutions were prepared by placing 0.5 ml of normal saline in standard agglutination tubes and transferring 0.5 ml of the diluted specimen serially from one tube to the next. Mixing was performed by drawing up and expelling the fluid vigorously with the transferring pipette. If centrifugation was performed on the first dilution the remaining 0.5 ml was carefully transferred to an empty agglutination tube without disturbing the cellular material in the bottom of the tube used for centrifugation.

Controls consisted of similar dilutions of a standard anti- S. seftenberg serum, serial dilutions of media, and a saline control. The titrations were accepted only if the media and saline controls demonstrated no agglutination.

Antigen was prepared and suspended in saline as described under MATERIALS AND METHODS. Each of the tubes received 0.2 ml of the antigen suspension, and they were mixed by shaking. They were then incubated for 1 hour at 37° C and read by grossly viewing the suspended bacteria for agglutination. Final readings were made after allowing the tests to remain over night at 4° C. The final end point was taken as the last tube showing definite evidence of agglutination of the bacteria as they were gently resuspended by making the fluid slowly

swirl in the tubes.

The standard sera could be consistently titrated to within one tube of the measured value. Thus there was an approximate variation of three tubes, and the values recorded did not establish *in vitro* antibody synthesis.